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Analysis of Haemoglobin Variants Using Immobilized pH Gradients[1])

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Summary: We present a new method for analysis of haemoglobin variants in immobilized pH gradients. The isoelectric fractionation is performed in polyacrylamide gels with copolymerized pH gradients between pH 7.0 and 8.0. The common haemoglobin variants (HbA, HbF, HbS, HbC, HbE and HbA_2) are clearly resolved within this pH range. The high resolving power and reproducibility of immobilized pH gradients combined with extremely short separation times make this fractionation technique superior to all electrokinetic procedures used for haemoglobin analysis. At present, isoelectric focusing in immobilized pH gradients is a complementary tool for haemoglobin analysis when classical electrophoretic techniques do not provide sufficient resolution. We hope that in the future this technique will gain the popularity it deserves.

Introduction

Most clinical chemistry laboratories favour classical electrophoretic techniques (cellulose acetate or citrate agar gel electrophoresis) for haemoglobin analysis. The use of carrier ampholyte-generated pH gradients (CA-IEF) is restricted to few specialized laboratories. This powerful separation technique, introduced in the 1960's (1), allowed the separation of the common haemoglobin variants encountered, i.e. HbA ($\alpha_2\beta_2$), HbF ($\alpha_2\gamma_2$), HbS (β6 Glu → Val) and HbA_2 ($\alpha_2\delta_2$). Separation of pathological haemoglobins such as HbH (β_4) and *Bart*'s haemoglobin (γ_4) never created any problems. Separation of electrically neutral mutants, however, was not possible in all cases. Nevertheless, under appropriate conditions, e.g., using chemical spacers, HbE (β26 Glu → Lys) and HbC (β6 Glu → Lys) could just be separated from HbA_2. Excellent description of CA-IEF and evaluation of the results can be found in the reviews l.c. (2–4).

The separation of haemoglobin variants was considerably improved with the introduction of immobilized pH gradients (5). *Righetti* and his group have built the basis for haemoglobin fractionation in immobilized pH gradients including the separation of "electrophoretically silent" variants (Hb-San Diego, Hb-Beirut and HbF-Sardinia), of globin chains (G-γ and A-γ), of animal haemoglobin mutants (from cattle, sheep, dog and mouse) and even the separation of tryptic digests of the α and β chains (6–8). Amazingly, despite the excellent separations obtained using immobilized pH gradients, no protocol for routine haemoglobin electrophoresis has been published so far.

Immobilized pH gradients are created using non-amphoteric acrylamido buffers having defined pK_a values. These buffers are copolymerized within the polyacrylamide matrix thus yielding a defined pH gradient. Thus, the pH gradient exists before isoelectric focusing, as opposed to CA-IEF where the pH gradient is established during the electrophoretic run. Furthermore, immobilized pH gradients do not exhibit gradient instability and cathodic drift that were the Achilles' heel of CA-IEF (9).

[1]) Results leading to this publication were presented at the 1991 meetings of the Deutsche Gesellschaft für Laboratoriumsmedizin, Frankfurt and the Deutsche Gesellschaft für Klinische Chemie, Nürnberg (12, 13).

We present a protocol for the analysis of haemoglobin variants using immobilized pH gradients in which we have attempted to standardize both the gel-casting and the electrophoretic procedure. Highly reproducible immobilized pH gradients can be generated using computer-driven burettes (10, 11). For isoelectric focusing, the semi-automated PhastSystem was used. Summaries of the procedure have already been published (12, 13). Using our protocol, the separation time is reduced to barely 45 minutes. Since only two commercially available acrylamido derivatives are required, we hope that this method will be recommended for international standardization that has (especially for conventional isoelectric focusing) been painfully missed.

Materials and Methods

Acrylamide, methylenebisacrylamide, tetramethylethylenediamine (TEMED) and ammonium persulphate were obtained from Bio-Rad Laboratories, Richmond, CA, USA. Acrylamido buffers, Gel-Bond supporting foils, carrier ampholytes between pH 5.0 and 8.0, glass plates for the preparation of thin polyacrylamide gels as well as the PhastSystem were from Pharmacia Biosystems, Stockholm, Sweden. All other chemicals of the highest purity grade were purchased from Merck, Darmstadt, Germany. The computer-driven burettes linked with an Apple I computer were obtained from Desaga, Heidelberg, Germany. Modern versions of burettes can be used in combination with IBM compatible personal computers. The burettes can be purchased from Schott, the piston pumps and the mixing chamber from Desaga and the software from Prof. *Altland* (Institute of Human Genetics, Gießen, Germany).

Sample preparation

EDTA-blood was obtained from patients after phlebotomy in a supine position. Sample pretreatment was performed as recommended by the International Committee for Standardization in Haematology (14). Briefly, one volume of blood was washed 3 times in saline solution and lysed with 1.5 volumes of CCl_4 and H_2O in a ratio 2 : 1. After centrifugation at 3000 min^{-1} in a table-top centrifuge, the upper aqueous phase was retrieved. 100 µl of a KCN solution (1.5 g/l) was added to 1 ml of the haemolysate. Carbon monoxide was bubbled through the samples for 5 min after which the samples were aliquot and stored frozen at −80 °C.

Gel Preparation

Immobilized pH gradients were prepared using the following recipe (9):

	Acidic solution pH 7.0	Basic solution pH 8.0
Acrylamido buffer pK 7.0	102 µl	177 µl
Acrylamido buffer pK 3.6	9 µl	16 µl
T30C4*	666 µl	666 µl
Glycerol (870 g/l)	888 µl	–
TEMED	2 µl	2 µl
Persulphate (400 g/l)	4 µl	4 µl
H_2O ad	3500 µl	3500 µl

Ten 0.5 mm thin gels were prepared using computer-driven burettes and polymerized at 50 °C for 1 h (15). They were then washed three times for 20 min each in distilled water, for a further 15 min in 2 g/dl glycerol and finally air-dried. The gel size obtained with the total volume listed above (7.0 ml) was 250 × 50 × 0.5 mm. Five gels for the PhastSystem can thus be obtained (50 × 50 × 0.5 mm). On each gel at least 8, in most cases 12 samples can be analyzed. In this manner, enough gels for the analysis of up to 600 patients can be prepared simultaneously and stored at −20 °C where they are stable for at least 12 months (9).

Electrophoretic conditions

Before use, the gels were rehydrated in 1 g/dl carrier ampholyte (pH 5.0–8.0) solution for 1 h (16, 17). The gels were then placed on the cooling block of the PhastSystem where the temperature was set at 10 °C. 1 µl of each haemolysed sample was applied for electrophoresis at the anodic end of the pH gradient. The electrodes had direct contact with the gels in the PhastSystem. The electrophoresis unit was programmed as follows:

Sample appl down at 1.1				0000 vH
Sample appl up at 1.2				0050 vH
Step 1.1	300V	5mA	5W	0050 vH
Step 1.2	2000V	5mA	5W	1000 vH
Extra alarm to sound at 1.2				0995 vH

The electrophoresis time was approximately 45 min after which the gels were dried without prior fixation. This procedure maintains the brilliant red colour of the haemoglobin variants, which can be clearly distinguished from the brownish hue of the oxidized products.

Results

Figure 1 shows the composite picture of four electrophoretic runs to demonstrate the quality and reproducibility of the separations. The haemoglobin variants have been labelled A, F, S, A_2, and C omitting the prefix Hb. Thus A stands for HbA, F stands for HbF and so forth. In all gels, the anode is uppermost.

Gel 1 shows commonly encountered variants. In the first lane from the left HbA and HbS are shown. Lane two shows the results obtained from haemolysate of foetal cord blood demonstrating the presence of HbA and HbF and some minor anodic bands corresponding to non-pathological acetylated HbF variants. In the pattern from the other three samples, HbA, HbA_2 and HbA_{1c} can be recognized. Note that HbF is completely resolved from HbA, a problem frequently encountered in cellulose acetate gels.

In gel 2, the first three lanes are identical to those in gel 1. In the lane marked with an arrow, the pattern

* T30C4 refers to the acrylamide stock solution. T = 30 refers to the total amount of acrylamide and bisacrylamide (in percent), i.e., 30 g/dl. C = 4 refers to the fraction of the crosslinker bisacrylamide (in percent) present in the solution. This means that 100 ml of the T30C4 solution contain 1.2 g bisacrylamide and 28.8 g acrylamide.

obtained from the haemolysate of a patient with heterozygous β-thalassaemia is shown. Note the sharp HbA_2 band obtained at the basic end of the pH gradient.

In gel 3, haemolysate from a neonate was subjected to isoelectric focusing. In this sample, not only HbA and HbF, but also an additional variant (marked with an arrow), slightly more basic than HbF can be recognized. This variant was identified to be HbF-Sardinia. This γ-chain variant could not be detected about six months after birth, thus confirming the diagnosis of HbF-Sardinia.

In gel 4, we show that at the basic end of the pH gradient, HbC can be completely resolved from HbA_2. In the last lane of gel 4 (also marked with an arrow) the pattern from a patient with homozygous sickle cell anaemia is shown. In this case, HbF, HbS and HbA_2 are seen. HbA cannot be detected (also compare with gel 3, lane 3 from the left). The other samples in all four gels are from routine electrophoretic runs demonstrating that all variants can easily be recognized.

Discussion

Isoelectric focusing, although it has definite advantages, has not been extremely popular for the analysis of haemoglobinopathies. Two recent leading textbooks in the field of laboratory medicine, one in English and the other in German, stay very reserved towards this technology (18, 19). One reason for this reserved attitude may be that most laboratories are accustomed to conventional electrophoretic techniques such as cellulose acetate or agarose gels. They are easy to handle, are not time-consuming and do not require specialized instruments. The buffers and matrices required for such electrophoretic runs are provided by a number of manufacturers.

On the other hand, gel preparation for isoelectric focusing is a complicated procedure. With the technology now available, however, we believe that these problems are finally over. Preparation of immobilized pH gradients can be performed using computer-driven burettes. Assuming that the limiting solutions are prepared precisely, the quality of the gels is only dependent on the computer, which always creates reproducible pH gradients. Immobilized pH gradients can, however, be prepared without specialized instrumentation. In these cases, gradient gels can be prepared using a two-vessel gradient mixer.

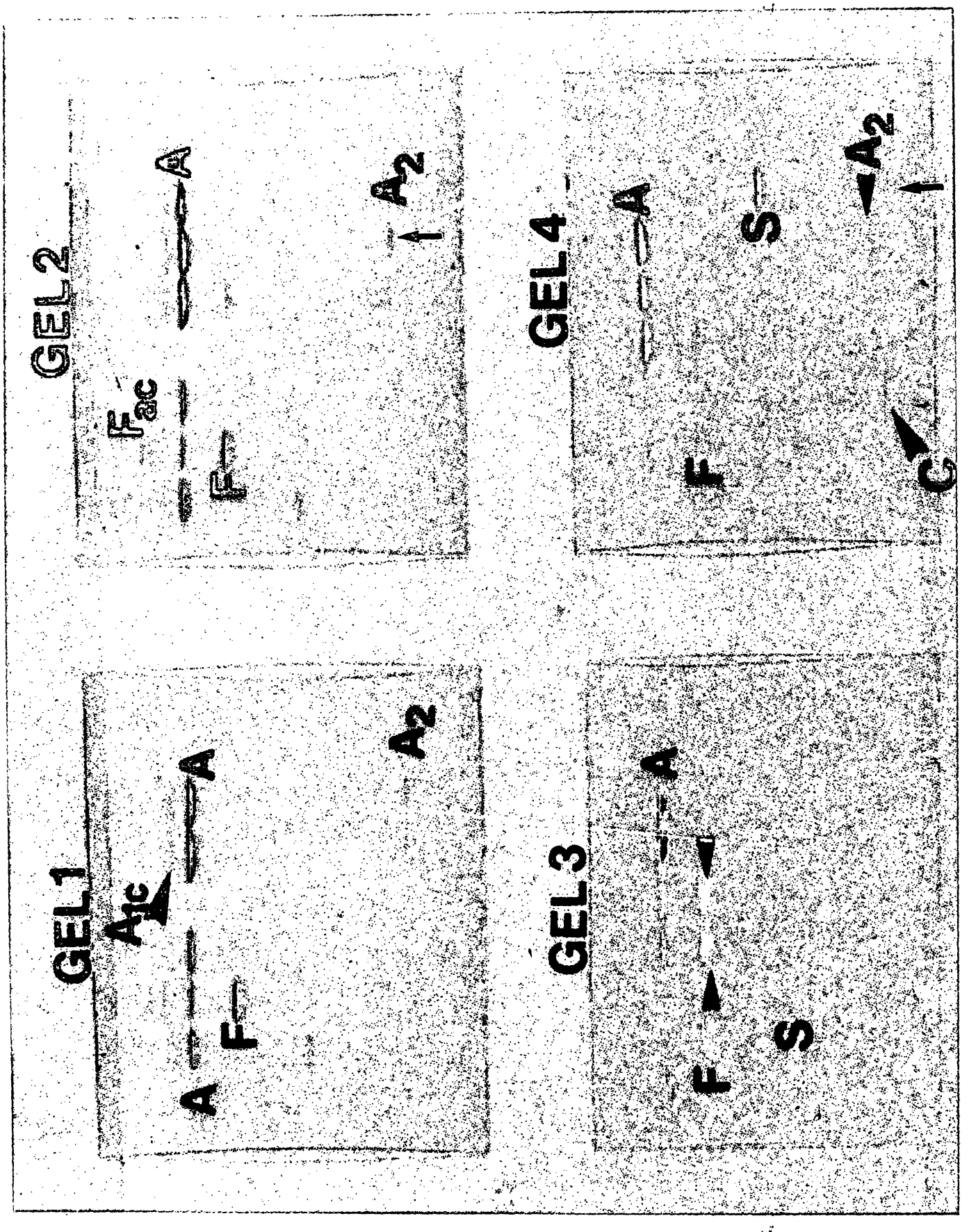
An optimal sample preparation is of utmost importance. Since the separations in pH gradients are only charge dependent, any charge differences (e.g. oxidation state of iron Fe^{2+} or Fe^{3+}) will give rise to additional bands that may confound the interpretation of the results (20). In our experience, the protocol recommended by the International Committee for Standardization in Haematology fulfills all demands for excellent sample preparation, although (as one of the reviewers has correctly pointed out) some haemoglobin variants (HbM) react slowly with KCN.

The resolving power of immobilized pH gradients is in the magnitude of ΔpI of 0.001 pH units (21). We demonstrate that this high resolution is maintained in our system with two known haemoglobin variants.

HbF-Sardinia is a neutral (γ75 isoleucine to threonine exchange) haemoglobin mutant. Immobilized pH gradients were shown to be capable of resolving HbF from HbF-Sardinia (7, 8) in ultra-narrow pH gradients spanning 0.22 pH units over an inter-electrode distance of 10 cm. The course of the pH gradient corresponds to 0.022 pH units/cm. We show that complete resolution of these variants is possible in our system (0.25 pH units/cm, i.e., in a pH gradient that is ten times more steep). Similar results have been obtained for HbC and HbA_2. This demonstrates that practically all known haemoglobin mutations can, in fact, be recognized in our system.

Thus, the advantages of our electrophoretic system for the diagnosis of haemoglobinopathies using immobilized pH gradients can be summarized as follows:

1. Only two chemicals (acrylamido buffers with pK_a of 7.0 and 3.6) are used to generate the pH gradient instead of carrier ampholytes that may contain a multitude of different species (21). This should enable international standardization of the analytical procedure.
2. A number of gels can be prepared simultaneously and stored. Depending on the number of samples to be analyzed, pieces of the gel can be cut and rehydrated in the appropriate buffer before electrophoresis.
3. Separation times using the PhastSystem are extremely short. This makes immobilized pH gradients a clear-cut alternative to standard electrophoretic procedures used in most laboratories (cellulose acetate or agar gel electrophoresis). It should be noted, however, that the PhastSystem is not mandatory. Any laboratory equipped with a flatbed electrophoresis chamber can use our protocol and maintain short separation times.
4. Immobilized pH gradients are practically indefinitely stable and thus offer high reproducibility of band positions from run to run.


GEL 1
A1c
A
A2
A
F
GEL 2
Fac
A
A2
F
GEL 3
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S
GEL 4
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S
A2
F
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5. The relatively shallow gradient used for fractionation (0.25 pH units/cm) maintains the high resolving power that is not matched by any other electrophoretic technique.

In summary, we maintain that the use of immobilized pH gradients in combination with the PhastSystem represents an extremely reproducible low-cost, high resolution and high efficiency system for haemoglobin anaylsis. Presently, the use of immobilized pH gradients is restricted to a few specialized laboratories. We hope that this technique achieves more popularity in the future.

Notes Added During Revision of the Manuscript

We would like to comment on the statement of one of the reviewers that with exception of HbH-disease, Hb *Bart*'s syndrome, Lepore variants and Hb Constant Spring anomalies, thalassaemias and hereditary persistence of HbF are not diagnosed using electrophoretic techniques.

To clear misunderstandings, the aim of this paper was to present the potentialities of immobilized pH gradients for the diagnosis of haemoglobinopathies. This does not mean that electrophoresis is the only method used by us. For the accurate diagnosis of haemoglobin disorders, supplementary tests such as blood picture, microscopic examination of blood smears, haptoglobin quantification and measurement of HbA_2 and HbF concentrations are mandatory.

We think that the quantification of HbA_2 using ion-exchange chromatography alone is not sufficient for the diagnosis of β-thalassaemia, since too many haemoglobin variants (HbS, HbC, HbE, HbO-Arab and others) interfere with accurate determinations. Therefore, we use electrophoretic analysis as an adjunct.

The same argument is valid for the diagnosis of hereditary persistence of HbF. The heterozygous form of this disorder demonstrates HbF fractions of up to 20% of total haemoglobin. In the homozygous form, HbF may constitute the total haemoglobin fraction. The question here is – which is the best and accurate method for HbF quantification? One way is the measurement of alkali-resistant haemoglobin. The other is the densitometric evaluation of HbF after electrophoresis. We have shown that HbF focuses as a clean band in immobilized pH gradients. Furthermore, *Righetti* et al. have demonstrated that the detection limit of single haemoglobin fractions in immobilized pH gradients is 0.5% (9). Thus, we prefer using electrophoresis and densitometry as opposed to the more cumbersome methods for quantification of alkali-resistant haemoglobin.

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Fig. 1. Composite picture of four electrophoretic runs for separation of haemoglobin variants in immobilized pH gradients between pH 7.0 and 8.0. The gels were prepared using two acrylamido derivatives. The pK 7.0 derivative was the buffering ion and the pK 3.6 the titrant, with which the limiting solutions were adjusted to pH 7.0 and 8.0. The acidic solutions was rendered dense with glycerol after which the gradients were prepared using computer driven burettes. The gels were washed for 1 h, dried and stored at −20 °C. Before use, the gels were rehydrated in carrier ampholytes as described in Materials and Methods. 1 µl of each haemolysate was subjected to isoelectric focusing, which lasted roughly 45 min. The electrophoresis conditions are described in Materials and Methods. After isoelectric focusing, the gels were dried with a hair-dryer without staining. In all gels, the anode, corresponding to pH 7.0 is uppermost. The lower gel end corresponds to pH 8.0. The haemoglobin variants are marked A, A_{1c}, F, S, A_2 and C omitting the prefix Hb. Further explanations of the samples are supplied in the text.

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